

## Resistance and Virulence of *Pseudomonas aeruginosa* Clinical Strains Overproducing the MexCD-OprJ Efflux Pump<sup>▽</sup>

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Since their initial description 2 decades ago, MexCD-OprJ-overproducing efflux mutants of *Pseudomonas aeruginosa* (also called *nfxB* mutants) have rarely been described in the clinical setting. Screening of 110 nonreplicate clinical isolates showing moderate resistance to ciprofloxacin (MIC from 0.5 µg/ml to 4 µg/ml) yielded only four mutants (3.6%) of that type harboring various alterations in the repressor gene *nfxB*. MexCD-OprJ upregulation correlated with an increased resistance to ciprofloxacin, cefepime, and chloramphenicol in most of the clinical strains, concomitant with a higher susceptibility to ticarcillin, aztreonam, imipenem, and aminoglycosides. Evidence was obtained that this increased susceptibility to aminoglycosides results from the impaired activity of efflux pump MexXY-OprM. Furthermore, MexCD-OprJ upregulation was found to impair bacterial growth and to have a strain-specific, variable impact on rhamnolipid, elastase, phospholipase C, and pyocyanin production. Review of patient files indicated that the four *nfxB* mutants were responsible for confirmed cases of infection and emerged during long-term therapy with ciprofloxacin. Taken together, these data show that, while rather infrequent among *P. aeruginosa* strains with low-level resistance to ciprofloxacin, MexCD-OprJ-overproducing mutants may be isolated after single therapy with fluoroquinolones and may be pathogenic.

Ten intrinsic multidrug efflux systems belonging to the RND (resistance nodulation cell division) family have been characterized so far in *Pseudomonas aeruginosa* (58). Of all these pumps, only MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN have been reported to provide significant resistance to antibiotics when stably overproduced upon mutations (see reference 53 for a recent review). In contrast to MexAB-OprM and MexXY-OprM, the MexCD-OprJ system does not contribute to the natural resistance of the pathogen to antimicrobials (54). However, alteration of the *nfxB* gene, whose product strongly represses the *mexCD-oprJ* operon, leads to a dramatic increase in MexCD-OprJ production and significant cross-resistance to fluoroquinolones, macrolides, and zwitterionic cepheims such as ceftiofur and cefepime (15, 35, 46, 51, 54). In addition, most but not all of the so-called “*nfxB* mutants” appear to be more susceptible than wild-type strains to aminoglycosides and other β-lactams (e.g., carbenicillin, aztreonam, and imipenem) (15, 24, 25, 45, 54). They also may exhibit variable levels of resistance to tetracycline and chloramphenicol, thus making the *NfxB* phenotype difficult to identify among clinical strains. Such changes in the *NfxB* phenotype have been attributed to variations in the levels of MexCD-OprJ production (45). More recently, the increased susceptibility of in vitro-selected *nfxB* mutants to β-lactams (except imipenem) was attributed to the decreased expression

of system MexAB-OprM and/or deficient drug induction of intrinsic β-lactamase AmpC (13, 47).

Since the first description of these mutants by Hirai et al. (15) 2 decades ago, very few *nfxB* strains have been identified in the clinical setting (24, 25, 26, 56, 62), thus suggesting that these mutants are either underrecognized because of their variable drug resistance phenotype or poorly pathogenic for humans. In support of the latter proposal, Linares et al. and Sanchez et al. (38, 57) recently reported that MexCD-OprJ overproduction is associated with reduced fitness, virulence, and type 3 secretion system (T3SS)-dependent cytotoxicity in laboratory *nfxB* mutants.

In an effort to better define the clinical relevance and the prevalence of this type of efflux mutants in *P. aeruginosa*, we investigated a large collection ( $n = 110$ ) of hospital isolates with reduced susceptibility to ciprofloxacin.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *Pseudomonas aeruginosa* PAO1, the genome of which has entirely been sequenced, was used as the reference wild-type strain throughout the study (23). EryR is a spontaneous *mexCD-oprJ*-overexpressing mutant of PAO1 selected on erythromycin (14). FE60 is a Δ*mexXY* derivative of PAO1 hypersusceptible to aminoglycosides (10). Plasmid pEXΔXYR (ticarcillin and gentamicin resistance), used previously to construct FE60, was employed here to inactivate genes *mexXY* in EryR (yielding mutant FK06), as described by El'Garch et al. (10). Two hundred five nonreplicate isolates of *P. aeruginosa* showing low-level resistance to ciprofloxacin (MICs ranging from 0.5 to 4 µg/ml) were collected between January and December 2006 from non-cystic fibrosis patients at the teaching hospital of Besançon (France). During this period of time, a total of 1,632 isolates of *P. aeruginosa* were isolated, of which 61.6% ( $n = 1,006$ ) were susceptible to ciprofloxacin, 1.8% ( $n = 29$ ) were intermediate, and 36.6% ( $n = 597$ ) were resistant according to the CLSI breakpoints (6). One hundred ten of the 205 strains with decreased susceptibility to ciprofloxacin (53.6%) were further randomly selected to analyze

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MexCD-OprJ pump expression. The four MexCD-OprJ-overproducing strains, 3308 (serotype O:6), 2439 (O:5), 2126 (O:6), and 1956 (O:2), identified in this study were found to be genotypically distinct by the random amplified polymorphic DNA method (data not shown) (44). Bacterial culturing was performed at 37°C in liquid (Mueller-Hinton broth [MHB]) or on solid (Mueller-Hinton agar [MHA]) medium with adjusted concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Becton Dickinson Microbiology Systems, Cockeysville, MD). Propagation of plasmid vector pMT45 (ticarcillin resistance) and its *nfxB* recombinant derivative pNF225 was achieved by adding 150 µg/ml ticarcillin to the media (52). Both plasmids were purified from strain PK1013E (52) and transferred into the *P. aeruginosa* strains by electroporation, as described by Choi et al. (4).

**Drug susceptibility testing.** The MICs of selected antibiotics were determined by the conventional serial twofold dilution method in MHA by using a Steers multiple inoculator and bacterial inocula of approximately  $10^4$  CFU per spot, as recommended previously (30). The inoculated plates were incubated at 37°C and examined visually after 18 and 24 h.

**Colony size.** Overnight cultures in MHB were serially diluted and spread on the surface of MHA in order to obtain 10 to 20 distinct colonies per plate after 24 h of incubation at 37°C. Five colonies of each *P. aeruginosa* strain were subsequently collected with the agar underneath and individually transferred to tubes containing 10 ml sterile saline buffer. Once serially diluted, these suspensions were used to inoculate MHA plates for CFU counting.

**Quantification of  $\beta$ -lactamase activity.** Induced and uninduced AmpC  $\beta$ -lactamase activities (nmol nitrocefin  $\cdot$  min $^{-1}$   $\cdot$  mg protein $^{-1}$ ) for strains PAO1, EryR, 3308, 2439, 2126, and 1956 were determined by spectrophotometric testing of crude French press lysates, as previously described (18). Measurements were done in triplicate for each strain. Variations in the results from one experiment to another were less than 15%. Induction of AmpC  $\beta$ -lactamase activity was obtained by the addition of 50 µg/ml cefoxitin to the MHB medium 3 h prior to the end of incubation. It should be mentioned that preliminary isoelectrofocusing experiments performed on the crude lysates revealed only the presence of a faint band at a pI of  $\geq 8.5$ , corresponding to AmpC in the selected strains (18).

**Quantitative real-time PCR.** The expression levels of operons *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, *mexGH-opmD*, *mexJK*, *mexVW*, and *mexXY* were assessed by reverse transcription-real-time PCR (RT-PCR) with the fluorescent dye Sybr green (Qiagen Sciences, MD) in a RotorGene RG3000 apparatus (Corbett Research, Sydney, Australia), essentially as described by Dumas et al. (9). The primers used for the amplification of genes *mexB* (MexB1, MexB2), *mexC* (MexC3, MexC4), *mexE* (MexE4, MexE5), *mexG* (MexG1, MexG2), *mexJ* (MexJ1, MexJ2), *mexV* (MexV1, MexV2), and *mexY* (MexY1A, MexY1B) have been reported elsewhere (9, 20, 27, 37). The gene expressions were normalized to that of the housekeeping gene *uvrD* (28). PAO1 was used as the wild-type control (values set at 1) and well-characterized gain-of-efflux derivatives as positive controls: PT629 for MexAB-OprM, EryR for MexCD-OprJ, PAO7H for MexEF-OprN, Mut-GR1 for MexXY, and PAO318 for MexJK (20). Induction of the *mexXY* operon in the *P. aeruginosa* strains and their transformants harboring either plasmid pMT45 or pNF225 (ticarcillin resistance) was achieved by adding chloramphenicol or gentamicin at one-half the MIC to the bacterial cultures for 4 h (27). Comparison of CFU counts on drug-free MHA and on MHA supplemented with ticarcillin at the end of the induction period in each experiment showed that more than 95% of the bacterial population still harbored pMT45 or pNF225 in the absence of selective pressure for plasmid maintenance. The RT-PCR data reported in the text are means of at least three independent determinations, with experiment-to-experiment variations being lower than 10%.

**DNA methods.** Plasmid DNAs were purified by using the Wizard Plus SV Minipreps kit (Promega France, Charbonnière, France) according to the manufacturer's protocol. To search for possible mutations, the *nfxB* gene was amplified with the pair of primers *nfxB1* (5'-ACGCGAGGCCAGTTTCT-3') and *nfxB2* (5'-ACTGATCTCCGAGTGTCG-3') and then sequenced on both strands by using an AB Prism 3130 genetic analyzer (Applied Biosystems, Courtabouef, France) at the Institut Fédératif de Recherche IFR133, Besançon, France. The quinolone resistance-determining regions of genes *gyrA*, *gyrB*, *parC*, and *parE* were amplified and sequenced in the clinical strains, as reported by Llanes et al. (40). All the quinolone resistance-determining region sequences turned out to be identical to that of wild-type strain PAO1. Amplification of *exoU* and *exoS* genes was performed as described previously (3).

**In vitro selection of *nfxB* mutants.** One-step spontaneous mutants were selected on MHA containing 1 µg per ml ciprofloxacin, from three genotypically different, drug-susceptible strains of *P. aeruginosa*, including the reference strain PA14 (55) and two previously characterized bacteremic isolates (3). The selective plates were inoculated with ca.  $10^8$  log phase bacteria and incubated at 37°C for 24 h. Ten resistant clones from each strain were analyzed by RT-PCR to identify those overexpressing gene *mexC*. Alteration of gene *nfxB* in the selected clones

was subsequently confirmed by nucleotide sequencing. Mutants KJ0707 (from PA14) and KJ0711 (from 19.1) were found to contain nonsense mutations in *nfxB* (A344G and T241C, respectively), while mutant KJ0705 (from 4.2) appeared to harbor a 8-bp deletion from position 137 to 144. In parallel, a new *nfxB* mutant of strain PAO1, named KJ0702, exhibiting a nonsense mutation (T154C) in *nfxB*, was isolated under the same conditions.

**Immunodetection of MexY and MexB.** Bacterial membranes were isolated, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by Western blotting with MexY- and MexB-specific polyclonal antisera (diluted 1:20,000 and 1:1,000, respectively), as already reported (22).

**Phenotypic assays.** Elastase activity was determined by an elastin Congo red (ECR) assay (61) of supernatants of cultures grown for 7 h in PB medium (11). Briefly, 50 µl of supernatant was added to 0.95 ml of 0.1 M Tris (pH 7.4) and 1 mM  $\text{CaCl}_2$  with an excess of ECR (4-mg/ml reaction volume; Elastin Company, Owensville, MO). After 18 h of incubation at 37°C, samples were centrifuged and degradation of ECR was determined by measuring absorbance at 495 nm in the supernatant. Elastase activity was expressed as the ratio optical density at 495 nm ( $\text{OD}_{495}$ )/ $\text{OD}_{600}$  of the culture.

Production of rhamnolipid was assessed on square-well plates (59) based on M9 minimal medium in which  $\text{NH}_4\text{Cl}$  was replaced by 0.1% glutamate, and supplemented with 0.2% glucose and  $\text{MgSO}_4$  (2 mM final concentration). Three-microliter droplets containing ca.  $10^6$  CFU were placed at equal distances on the dried plates, which were incubated for 24 h at 37°C and then for 24 h at 25°C. The rhamnolipid production was estimated semiquantitatively by measuring the diameter of the blue halos forming around bacterial spots.

Activity of phospholipase C in the supernatants of stationary-phase cultures was quantified spectrophotometrically by using NPPC (*p*-nitrophenyl-phosphorylcholine) as a chromogenic substrate, according to the method developed by Berka et al. (2). Briefly, 2 ml of tryptose minimal medium was inoculated with 200 µl of a bacterial suspension calibrated at a McFarland standard of 0.4 in saline buffer. After 24 h of incubation at 37°C, the culture supernatant was recovered and treated with 10 mg of decolorizing carbon. Ten-microliter fractions of medium were then added to 90 µl of a reaction mixture containing 250 mM Tris-HCl (pH 7.2), 10 mM NPPC, 60% (wt/vol) glycerol, and 1 µM  $\text{ZnCl}_2$ . Absorbance at 405 nm of triplicate samples was recorded after 1 h of incubation at 37°C.

In addition to eliciting phospholipase C production, the low-phosphate tryptose minimal medium was found to elicit strong production of pyocyanin in the various strains of *P. aeruginosa*. The redox active dye in the supernatants of 24-h cultures was quantified spectrophotometrically, as described by Essar et al. (11). Briefly, 1-ml fractions of supernatant were extracted twice with 1 ml chloroform, and pyocyanin was reextracted from the solvent in 1 ml of 0.2 HCl. Absorbance of this solution was measured at 520 nm.

**LDH release assay.** The T3SS-dependent cytotoxicity of the strains was assayed with the murine macrophage cell line J774 (ATCC), grown in Dulbecco's modified Eagle medium (Gibco) supplemented with heat-inactivated fetal calf serum (Gibco) at 10%. The cells were seeded in 24-well culture plates at  $3 \times 10^5$  cells per well 20 h before infection. The bacterial strains were grown overnight in Luria broth, diluted to an  $\text{OD}_{600}$  of 0.1, and grown for an additional 3 h to an  $\text{OD}_{600}$  of approximately 1.0. Macrophages were infected with bacteria at a multiplicity of infection of 5. Cytotoxicity was assessed at 2 h and 3 h postinfection by determination of lactate dehydrogenase (LDH) release into infected supernatants using a cytotoxicity detection kit (Roche), as described previously (7). The 100% value represented the LDH released from uninfected cells lysed with Triton X-100.

**Clinical data.** Strain 3308 was isolated from a 70-year-old woman with long-term history of acute myeloid leukemia and admitted to the hospital for pneumonia of unknown origin. The strain was recovered from the sputum and a single blood culture after 32 days of hospitalization and multiple courses of chemotherapy, including a 9-day per os treatment with ciprofloxacin (1.5 g per day) and cefpodoxime (400 mg per day). The patient's death, which occurred 1 month later despite administration of adequate antipseudomonal antibiotics (2 g per day ceftazidime and 750 mg per day amikacin for 19 and 3 days, respectively), was caused by a septic shock not documented bacteriologically, concomitant with unfavorable evolution of her leukemia. Strain 2439 was isolated from a purulent surgical wound in a 39-year-old male admitted to the hospital for multiple trauma, after 11 days of single therapy with intravenous ciprofloxacin (1 g per day). The patient's fatal outcome 1 month later was not attributed to *P. aeruginosa*. Strain 2126 was isolated from a 51-year-old outpatient male with severe external otitis, after a 10-day intravenous course of ciprofloxacin (800 mg per day) and topical application of ofloxacin. The clinical evolution was favorable, and the patient recovered from his infection. Finally, strain 1956 was recovered from a 65-year-old man admitted to the hospital for cerebrovascular atheroscle-

rotic disease. This patient was transferred 48 days after admission to the medical intensive care unit and intubated because of obstructive bronchitis. The *nfxB* mutant was isolated in a tracheal aspirate after 11 days of ciprofloxacin (400 mg per day) and ceftriaxone (1 g per day). The patient improved under treatment and was transferred to a tertiary-care center, where he died 1.5 month later from severe sepsis and pneumonia not documented bacteriologically.

**Nucleotide sequence accession numbers.** The GenBank accession numbers of *nfxB* sequences of strains 3308, 2439, 2126, and 1956 are EU525876, EU525877, EU525878, and EU525879, respectively.

## RESULTS

**Isolation of clinical *nfxB* mutants.** In vitro-selected MexCD-OprJ-overexpressing mutants have been reported to exhibit 4- to 16-fold increases in resistance to fluoroquinolones, macrolides, and zwitterionic cephalosporins (e.g., cefpirome and cefepime) compared to wild-type susceptible strains (14, 15, 24, 25, 45, 54). However, the NfxB phenotype may also include a 2- to 8-fold-higher resistance to tetracycline and chloramphenicol concomitant with a 2- to 32-fold-greater susceptibility to aminoglycosides and some  $\beta$ -lactams (e.g., ticarcillin, aztreonam, and imipenem) (15, 24, 45, 46), making its identification rather difficult in clinical strains. To get an insight into the prevalence of *nfxB* mutants in our hospital, we screened a collection of 110 nonreplicate non-cystic fibrosis clinical isolates showing low-level resistance to ciprofloxacin (MIC from 0.5 to 4  $\mu$ g/ml), compatible with expression of an efflux-based mechanism. Only four strains (3308, 2439, 2126, and 1956) appeared to overexpress the *mexC* gene significantly by RT-PCR (Table 1). Sequencing of the *nfxB* gene, whose product normally downregulates the *mexCD-OprJ* operon (54), confirmed that these genotypically distinct strains were *nfxB* mutants and revealed various nucleotide deletions (strains 3308, 2439, and 2126) as well as a missense mutation resulting in an Ala38 $\rightarrow$ Val substitution in the DNA binding domain (helix-turn-helix motif) of the NfxB protein (strain 1956) (51). The in vitro-selected *nfxB* mutant EryR (14) was found to harbor a nonsense mutation leading to a truncated NfxB peptide. Loss of function of NfxB has previously been associated with frameshift mutations (5, 8, 54) or disruption of gene *nfxB* by insertion sequences (8), as well as amino acid substitutions lying within (Leu40 $\rightarrow$ Gln, Arg42 $\rightarrow$ Gly, Arg42 $\rightarrow$ His) or outside (Arg82 $\rightarrow$ Leu, His87 $\rightarrow$ Arg, Leu88 $\rightarrow$ Pro) the helix-turn-helix domain of the protein (5, 26, 34, 51, 54). Of particular interest here was the observation that *mexC* expression in strain 1956 was somewhat lower (from 1.5- to 3.5-fold) than that in strains with disrupted *nfxB* genes, suggesting that the Ala38 $\rightarrow$ Val substitution might only partially compromise the ability of NfxB to bind to the promoter region of *mexCD-oprJ* and repress its expression. Consistent with this assumption, sequencing of the intergenic region between *nfxB* and *mexCD-oprJ* did not reveal additional alterations in that strain. The involvement of other Mex pumps in addition to MexCD-OprJ in the resistance of the clinical strains was ruled out by RT-PCR experiments that showed that the expression levels of genes *mexB*, *mexE*, *mexG*, *mexJ*, *mexV*, and *mexY* were similar in the four clinical *nfxB* mutants and PAO1 (data not shown except for *mexB* and *mexY*; Table 1).

**Drug resistance phenotypes of *nfxB* strains.** As shown in Table 2, strains 3308, 2439, and 2126 were phenotypically similar to previously reported type B *nfxB* mutants (45) including

TABLE 1. Relative expression levels of genes *mexC*, *mexB*, and *mexY* in clinical MexCD-OprJ-overproducing strains<sup>a</sup>

Strain (plasmid)	<i>nfxB</i> mutation <sup>b</sup>	Expression <sup>c</sup> (fold change) of gene:				
		<i>mexC</i> <i>mexB</i>		<i>mexY</i> in strains		
				Uninduced	Induced with <sup>d</sup> :	
					GEN	CHL
PAO1	None	1	1	1	56.2	187.9
EryR	C <sub>199</sub> T <sup>e</sup>	43.3	1.4	6.4	53.5	129.3
EryR(pNF225)		4.8	1.1	4.5	65.3	185
3308	$\Delta$ A <sub>122</sub> -G <sub>131</sub>	38.4	0.4	$\leq$ 0.1	0.2	1.1
3308(pNF225)		6.3	0.6	0.1	0.5	0.6
2439	$\Delta$ G <sub>177</sub> -A <sub>178</sub>	32.4	0.8	2.0	20.3	129.8
2439(pNF225)		7.8	0.7	0.4	19.3	117.4
2126	$\Delta$ C <sub>114</sub> -T <sub>130</sub>	74.9	0.9	1.1	10.6	101.8
2126(pNF225)		10.4	0.9	0.5	16.2	81.6
1956	C <sub>113</sub> T <sup>f</sup>	21.6	0.4	0.8	15.5	103.2
1956(pNF225)		6.4	0.2	0.7	33.5	122.4

<sup>a</sup> Control experiments showed that plasmid vector pMT45 had no influence on the RT-PCR results.

<sup>b</sup> Nucleotide positions are numbered according to the PAO1 *nfxB* sequence.

<sup>c</sup> Relative to the expression of uninduced strain PAO1 (which is assigned a value of 1). The presented values are means of three independent determinations.

<sup>d</sup> Bacteria were cultivated for 4 h in the presence of gentamicin (GEN) or chloramphenicol (CHL) at one-half the MIC.

<sup>e</sup> Nonsense mutation (Q<sub>67</sub> $\rightarrow$ amber stop).

<sup>f</sup> Missense mutation Ala<sub>38</sub> $\rightarrow$ Val. This strain also contains the transitions G<sub>62</sub>A (Arg<sub>21</sub> $\rightarrow$ His) and A<sub>167</sub>G (Asp<sub>56</sub> $\rightarrow$ Gly), present in wild-type reference strain PA14 (<http://v2.pseudomonas.com>).

EryR. Compared with wild-type strain PAO1, they exhibited some degree of resistance to ciprofloxacin (8-fold), cefepime (2- to 4-fold), and chloramphenicol (2-fold), with concomitant increased susceptibility to ticarcillin (4- to 8-fold), aztreonam (4-fold), imipenem (4- to 16-fold), and aminoglycosides (2- to 8-fold). As expected from the frameshift mutations found in *nfxB*, complementation of these bacteria with a plasmid-borne *nfxB* gene from PAO1 (named pNF225) restored wild-type levels of susceptibility to most drugs. Reminiscent of type A mutants, which produce lower levels of MexCD-OprJ than their type B counterparts (45), strain 1956 appeared to be less resistant to ciprofloxacin than strains 3308, 2439, and 2126 (fourfold increase in MIC; Table 2) and indistinguishable from PAO1 regarding its susceptibility to cefepime and imipenem. Also, consistent with operon *mexCD-oprJ* being partially derepressed in 1956, complementation of that strain with plasmid pNF225 indeed produced only 2- to 4-fold variations in the MICs of selected antibiotics compared with the 2- to 16-fold changes observed in strains 3308, 2439, and 2126. It should be mentioned that, whatever their A or B NfxB phenotype, all the clinical strains showed the same susceptibility as PAO1 to piperacillin, a poor substrate for MexCD-OprJ (data not shown) (49).

**Involvement of MexXY-OprM in the increased susceptibility to aminoglycosides.** The MexXY-OprM efflux system contributes efficiently to the natural resistance of *P. aeruginosa* to aminoglycosides under standard laboratory growth conditions

TABLE 2. Antibiotic susceptibilities of *P. aeruginosa* strains

Strain (plasmid)	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup> of:								
	CIP	FEP	CHL	TIC	ATM	IPM	GEN	TOB	AMK
PAO1	0.25	2	64	16	4	2	2	0.5	4
FE60	0.12	2	64	16	4	2	<u>0.25</u>	0.25	<u>1</u>
EryR	<b>2</b>	<b>8</b>	128	<u>2</u>	<u>1</u>	<u>0.5</u>	1	0.25	2
EryR(pNF225)	0.25	ND <sup>b</sup>	32	ND <sup>b</sup>	4	2	2	1	8
FK06	<b>2</b>	<b>8</b>	128	<u>4</u>	<u>1</u>	<u>0.5</u>	ND <sup>c</sup>	0.25	<u>1</u>
FK06(pNF225)	0.25	ND	64	ND	4	2	ND	0.25	<u>1</u>
3308	<b>2</b>	4	128	<u>2</u>	<u>1</u>	<u>0.12</u>	<u>0.25</u>	<u>0.12</u>	<u>0.5</u>
3308(pNF225)	0.12	ND	64	ND	4	2	1	0.25	<u>1</u>
2439	<b>2</b>	<b>8</b>	128	<u>4</u>	<u>1</u>	<u>0.5</u>	<u>0.5</u>	0.25	<u>1</u>
2439(pNF225)	0.25	ND	64	ND	4	2	2	0.5	2
2126	<b>2</b>	<b>8</b>	128	<u>4</u>	<u>1</u>	<u>0.5</u>	<u>0.5</u>	0.25	<u>1</u>
2126(pNF225)	0.25	ND	32	ND	4	1	2	0.5	2
1956	<b>1</b>	2	128	8	<u>1</u>	2	1	0.25	<u>1</u>
1956(pNF225)	0.25	ND	64	ND	4	4	2	0.5	2

<sup>a</sup> Modal values of three independent determinations. Underlined MICs are at least fourfold lower than those for wild-type strain PAO1; MICs in boldface are at least fourfold higher than those for wild-type strain PAO1. Abbreviations: CIP, ciprofloxacin; FEP, cefepime; CHL, chloramphenicol; TIC, ticarcillin; ATM, aztreonam; IPM, imipenem; GEN, gentamicin; TOB, tobramycin; AMK, amikacin. ND, not determined.

<sup>b</sup> Susceptibility to FEP and TIC could not be measured accurately in strains complemented with recombinant *nfxB* plasmid pNF225 because of the hydrolytic activity of plasmid-encoded  $\beta$ -lactamase toward these two molecules. Control experiments with parent vector pMT45 showed that addition of 150  $\mu\text{g/ml}$  ticarcillin to growth cultures for pNF225 maintenance did not influence the MICs of other antibiotics.

<sup>c</sup> This strain contains a chromosomal cassette conferring high-level resistance to gentamicin.

(1, 48). It has recently been demonstrated that these agents indirectly induce the expression of operon *mexXY* as a result of their interaction with the ribosome (27). Because the natural resistance to aminoglycosides requires the induced production of proteins MexX and MexY, we tested whether *mexXY* is still inducible in the *nfxB* mutants. Table 1 shows that both gentamicin and chloramphenicol at half the MIC were able to induce *mexXY* expression in strains EryR, 2439, 2126, and 1956. Additional RT-PCR experiments on genotypically different *nfxB* mutants selected in vitro on ciprofloxacin (KJ0702, KJ0707, KJ0705, and KJ0711 from strains PAO1, PA14, 4.2, and 19.1, respectively) confirmed these results (data not shown). In PAO1 and its *nfxB* mutant EryR, the drug-induced *mexY* levels were similar and not significantly influenced by complementation with *nfxB* plasmid pNF225. Interestingly, the uninduced transcription of *mexY* was found to be slightly higher in EryR and EryR(pNF225) than in PAO1, a result which correlated with an increased production of protein MexY in EryR (Western blotting data not shown). As in EryR, plasmid pNF225 had minor if any effects on basal or induced expression of *mexY* in strains 2439, 2126, and 1956. Similar results were obtained for 3308, with the notable difference that this strain expressed barely detectable, albeit still inducible, amounts of *mexY* mRNA. As confirmation that 3308 is strongly deficient in MexXY, the strain turned out to be as susceptible as  $\Delta$ *mexXY* mutant FE60 to aminoglycosides (Table 2). Sequencing experiments on strain 3308 did not reveal particular mutations in gene *mexZ* (whose product downregulates operon *mexXY*), in the *mexZ*-*mexXY* intergenic region, or in gene PA5471 (whose product is necessary for drug-induced expression of *mexXY* in strain PAO1) (50), a result which provides

indirect evidence for the existence of additional genes regulating *mexXY* expression.

Altogether these data clearly showed that the *nfxB* mutation has no influence on the expression levels of *mexXY* or the inducibility of *mexXY* by aminoglycosides. To further investigate the contribution of the MexXY-OprM system to the resistance of *nfxB* mutants to these antibiotics, we attempted to inactivate the *mexXY* operon with a gene replacement strategy (10). We were unsuccessful with the clinical strains, as the suicide plasmid pEX $\Delta$ XYR repeatedly integrated into the bacterial chromosome at sites other than the *mexXY* locus. In contrast, we could easily obtain a  $\Delta$ *mexXY* derivative from EryR (named FK06). Susceptibility to aminoglycosides of EryR was poorly affected by the inactivation of MexXY, suggesting that the activity of the pump is compromised as a result of MexCD-OprJ being upregulated (compare FK06 with EryR and FE60 in Table 2). Furthermore, the observation that FK06, once complemented with *nfxB* plasmid pNF225, exhibited unchanged susceptibility to these products, in contrast to EryR, strongly suggests that in *nfxB* mutants such as EryR, 2439, 2126, and 1956 the MexXY-OprM-mediated export of aminoglycosides is impaired. Interestingly, other investigators (60) also came to the conclusion that complex factors may influence the contribution of MexXY-OprM to aminoglycoside resistance in clinical strains.

**Contribution of MexAB-OprM to the susceptibility to  $\beta$ -lactams.** Transformation of EryR, FK06, 3308, 2439, 2126, and 1956 with plasmid pNF225 completely restored wild-type levels of resistance to aztreonam and imipenem (Table 2), thereby indicating that the increased susceptibility to  $\beta$ -lactams of all the *nfxB* mutants is linked to the alteration of

TABLE 3. Virulence of *nfxB* mutants

Strain	Rhamnolipid production <sup>a</sup>	Production (%) <sup>b</sup> of:			T3SS		Colony size (%) <sup>f</sup>
		Elastase <sup>c</sup>	Phospholipase C <sup>d</sup>	Pyocyanin <sup>c</sup>	Genotype	Cytotoxicity (%) <sup>e</sup>	
PAO1	++	100	100	100	<i>exoS</i>	79 ± 5	100
EryR	+	<b>60 ± 4</b>	125 ± 16	<b>57 ± 8</b>	<i>exoS</i>	72 ± 16	<b>35 ± 9</b>
PA14	++	296 ± 28	156 ± 26	89 ± 12	<i>exoU</i>	79 ± 4	73 ± 11
KJ0707	+	<b>222 ± 21</b>	<b>11 ± 4</b>	<b>36 ± 2</b>	<i>exoU</i>	75 ± 3	<b>32 ± 7</b>
4.2	++	160 ± 13	87 ± 6	148 ± 22	<i>exoS</i>	39 ± 3	107 ± 21
KJ0705	++	<b>31 ± 2</b>	78 ± 10	<b>33 ± 6</b>	<i>exoS</i>	33 ± 5	<b>23 ± 5</b>
19.1	++	191 ± 16	45 ± 12	152 ± 36	<i>exoS exoU</i>	77 ± 5	210 ± 37
KJ0711	±	198 ± 7	55 ± 6	<b>87 ± 7</b>	<i>exoS exoU</i>	76 ± 2	<b>49 ± 12</b>
3308	+	180 ± 13	91 ± 3	64 ± 11	<i>exoS</i>	70 ± 8	65 ± 17
2439	+	15 ± 4	95 ± 18	5 ± 3	<i>exoS</i>	84 ± 8	34 ± 13
2126	++	167 ± 12	68 ± 6	74 ± 6	<i>exoS</i>	71 ± 23	25 ± 6
1956	++	291 ± 25	36 ± 10	98 ± 13	<i>exoS</i>	47 ± 10	24 ± 7

<sup>a</sup> Semiquantitative estimation (from no production for an RhlR-negative mutant to the production level of PAO1 [++]) from triplicate samples. ±, standard deviation.

<sup>b</sup> Relative to PAO1 (100%). Values in boldface correspond to significant reduction in production in *nfxB* mutants compared with wild-type parents.

<sup>c</sup> Values are means of three independent experiments.

<sup>d</sup> Values are means of four independent experiments.

<sup>e</sup> Extracellular LDH activity at 3 h postinfection expressed as % of total lysis obtained with Triton X-100. Values are means of three experiments.

<sup>f</sup> Bacteria per colony relative to PAO1 (100%). Values are means of five determinations. Values in boldface correspond to significant reduction in colony size in *nfxB* mutants compared with wild-type parents.

*nfxB*. We therefore tested whether this phenotype is due to the reduced expression of efflux system MexAB-OprM and/or of chromosomally encoded  $\beta$ -lactamase AmpC, as previously reported for laboratory mutants (14, 47). All the *nfxB* mutants studied here, including KJ0702, KJ0707, KJ0705, and KJ0711 (data not shown), expressed gene *mexB* at comparable levels (from 0.4- to 1.4-fold that of PAO1), which remained unchanged upon complementation with pNF225 despite restoration of the wild-type susceptibility to  $\beta$ -lactams (Tables 1 and 2). Confirming these data, Western blot experiments revealed the presence of similar amounts of protein MexB in the mutants and their respective pNF225 transformants (not shown). Furthermore, the AmpC activities (uninduced versus induced with 50  $\mu$ g/ml cefoxitin) for PAO1 (13 versus 1,820 nmol nitrocefin min<sup>-1</sup> mg<sup>-1</sup> protein), EryR (29 versus 3,110 nmol nitrocefin min<sup>-1</sup> mg<sup>-1</sup> protein), 3308 (20 versus 2,670 nmol nitrocefin min<sup>-1</sup> mg<sup>-1</sup> protein), 2439 (19 versus 2,680 nmol nitrocefin min<sup>-1</sup> mg<sup>-1</sup> protein), 2126 (33 versus 5,140 nmol nitrocefin min<sup>-1</sup> mg<sup>-1</sup> protein), and 1956 (11 versus 4,550 nmol nitrocefin min<sup>-1</sup> mg<sup>-1</sup> protein) were roughly similar. Thus, in apparent contrast to the conclusions of other studies (14, 47), but reminiscent of our results with MexXY-OprM (see above), these data suggest that, while normally produced, efflux pump MexAB-OprM has partially lost its ability to efficiently transport  $\beta$ -lactam antibiotics in *nfxB* mutants.

**Pathogenicity of *nfxB* mutants.** Upregulation of efflux pumps has been reported to impact bacterial virulence in strain PAO1 (12, 33). For instance, MexAB-OprM- and MexCD-OprJ-overproducing mutants of PAO1 produce less pyocyanin, pyoverdine, casein protease, and elastase than their wild-type parent (12, 57); they also exhibit a reduced capacity to invade cell monolayers and to kill leukemic mice (16) or nematodes such as *Caenorhabditis elegans* (57). Recently, a MexCD-OprJ-overproducing derivative of PAO1 was found to be partially deficient in the T3SS, a needle-like

export machinery that allows direct injection of various cytotoxins into eucaryotic cells (38). Whether these partial virulence defects are clinically significant in human infections remains to be confirmed, as MexAB-OprM-upregulated strains are very prevalent in hospitalized patients and may be involved in severe infections (17, 20, 21, 39). In vitro assays showed that EryR, 3308, 2439, 2126, and 1956 differed quite significantly in terms of rhamnolipid, elastase, phospholipase C, and pyocyanin production, as well as in terms of T3SS-dependent cytotoxicity (compare 2439 and 1956 in Table 3). The effects of MexCD-OprJ upregulation on the virulence of *P. aeruginosa* also appeared to be variable and dependent on the genetic background of the strains (compare EryR to PAO1, KJ0707 to PA14, KJ0705 to 4.2, and KJ0711 to 19.1 in Table 3). All the *nfxB* mutants of the study produced smaller colonies on MHA than PAO1 or their respective wild-type parents (Table 3), strongly suggesting that when overproduced this pump represents a significant burden for *P. aeruginosa*. However, since strains 3308, 2439, 2126, and 1956 were involved in confirmed cases of infection (see Materials and Methods for more clinical data), it seems evident that some *nfxB* mutants may remain pathogenic, at least under certain clinical situations.

**In vivo selection of *nfxB* mutants.** Many studies have highlighted the fact that fluoroquinolones readily select for *nfxB* mutants in vitro (24, 25, 32, 46) as well as in infected animals (29, 31, 43). Although very few clinical *nfxB* strains have been characterized to date (24, 25, 62), the circumstances of their selection in vivo have rarely been documented (56). Review of the treatments administered to the patients with strains 3308, 2439, 2126, and 1956 clearly showed that these *nfxB* mutants emerged under long-term single therapy (as cefpodoxime and ceftriaxone are quite inefficient against *P. aeruginosa*) with fluoroquinolones (mostly ciprofloxacin), as in the case described by Reinhardt et al. (56). Whether these mutants evolved from initially susceptible strains or were

selected in the hospital environment by antiseptics such as triclosan (5) prior to infecting patients could not be determined precisely.

## DISCUSSION

Previous reports have pointed out the fact that the typical *NfxB* resistance phenotype (e.g., see EryR in Table 2) may be masked in clinical strains by other mechanisms such as the production of  $\beta$ -lactamase, the loss of porin OprD (24), or fluoroquinolone target alterations (62). Despite an elevated consumption of fluoroquinolones and high rates of gain-of-efflux mutants (MexAB-OprM, MexXY-OprM) in France (17, 21), this study identified only 3.7% *nfxB* mutants among a large collection of strains showing moderate resistance to ciprofloxacin (MIC from 0.5 to 4  $\mu$ g/ml). In support of the notion that such mutants are rather infrequent in the clinical setting except maybe in cystic fibrosis patients (26), another study failed to detect *nfxB* strains among 38 strains more resistant to cefepime than ceftazidime (a phenotype in agreement with MexCD-OprJ overexpression), which had been collected in two French hospitals (20). However, it could be argued that *nfxB* mutations may be more frequent in strains with high resistance to fluoroquinolones, as up-regulation of MexCD-OprJ is known to potentiate mutations in target genes such as *gyrA* (41). Of note, 29 of the 38 isolates reported in the latter study displayed levels of resistance to ciprofloxacin of  $\geq 2$   $\mu$ g per ml. More recently, no *nfxB* mutants were found among 120 bacteremic *P. aeruginosa* isolates, 11 and 36% of which were overproducers of MexAB-OprM and MexXY-OprM, respectively (17). Finally, only a single *nfxB* mutant could be identified in a French multicenter collection of 170 clinical strains, including 103 isolates with ciprofloxacin MICs of  $\geq 2$   $\mu$ g per ml (19).

What are the reasons for the relatively low prevalence of this type of efflux mutants in French hospitals? An attractive hypothesis could be that these bacteria are avirulent because of impaired fitness. The slow growth of these mutants likely represents a handicap for their development in the host in addition to variable defects in rhamnolipid, elastase, phospholipase C, and pyocyanin production. However, the pathogenicity of *P. aeruginosa* is multifactorial, strain specific, and dependent on complex host factors (36). Since the virulence defects detected in *nfxB* mutants are partly similar to those of MexAB-OprM overproducers (12, 16, 57), it would be simplistic to conclude that these changes are the sole elements accounting for the rare occurrence of *nfxB* mutants in patients (as *nalB* mutants are quite prevalent). Whether the four clinical *nfxB* mutants described here have specific virulence features or secondary mutations allowing them to compensate for virulence defects caused by the *nfxB* mutation is not known. Alternatively, the observation that in the four patients the development of MexCD-OprJ mutants occurred under single therapy with ciprofloxacin strongly suggests that a combined treatment with an aminoglycoside or a  $\beta$ -lactam antibiotic, as recommended for *P. aeruginosa* infections in France, would have prevented this emergence. Because of their higher susceptibility to aminoglycosides and  $\beta$ -lactams (except zwitterionic cephalosporins; Table 2), *nfxB* mutants are likely to be more readily eradicated by

these antibiotics. Thus, the facts that fluoroquinolones are rarely used alone to treat *P. aeruginosa* infections and that zwitterionic cephalosporins (e.g., cefepime and cefpirome) are not available in our hospital probably minimize the risk of selection of *nfxB* mutants.

At this point, the reasons for the apparent reduced activity of systems MexXY-OprM and MexAB-OprM in *nfxB* mutants are unclear. A plausible explanation would be that *P. aeruginosa* cannot energetically (e.g., proton motive force) or mechanically (e.g., pump assembly) sustain the functioning of both pumps when MexCD-OprJ is upregulated. The slow growth of *nfxB* mutants supports the notion that the resistance provided by the latter mechanism represents a significant burden for the cell. However, this assumption needs to be confirmed.

Our results are concordant with other data (24, 25) indicating that most of these mutants may tentatively be identified among clinical strains with low-level ciprofloxacin resistance (MIC  $\geq 1$   $\mu$ g/ml) if they satisfy to at least two of the following criteria: (i) an increased susceptibility to aminoglycosides (e.g., amikacin), (ii) an increased susceptibility to  $\beta$ -lactams (e.g., aztreonam), and (iii) a higher resistance to zwitterionic cephalosporins (e.g., cefepime) (Table 2). However, the use of RT-PCR or specific efflux inhibitors such as MC-272,457 (42) is still required to confirm *mexCD-oprJ* overexpression.

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